

PROTOCOL

Protocols for marker-free gene knock-out and knock-down in *Kluyveromyces marxianus* using CRISPR/Cas9

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^{*}Corresponding author: School of Microbiology, University College Cork, Cork T12 K8AF, Ireland. Tel: +353 87 219 6075; E-mail: j.morrissey@ucc.ie[†]Present address: Teagasc Food Research Centre, Moorepark, Fermoy, Cork P61 C996, Ireland**One sentence summary:** CRISPR protocols for easy genome engineering of the industrial yeast *K. marxianus*.**Editor:** Zongbao Zhao[‡]John P. Morrissey, <https://orcid.org/0000-0001-7960-2001>

ABSTRACT

There is increased interest in strain engineering in the food and industrial yeast *Kluyveromyces marxianus* and a number of CRISPR/Cas9 systems have been described and used by different groups. The methods that we developed allow for very rapid and efficient inactivation of target genes using the endogenous DNA repair mechanisms of the cell. The strains and plasmids that we use are freely available, and here we provide a set of integrated protocols to easily inactivate genes and to precisely integrate DNA fragments into the genome, for example for promoter replacement, allelic swaps or introduction of point mutations. The protocols use the Cas9/gRNA expression plasmid pUCC001 and Golden Gate assembly for molecular cloning of targeting sequences. A genome-wide set of target sequences is provided. Using these plasmids in wild-type strains or in strains lacking non-homologous end-joining (NHEJ) DNA repair, the first set of protocols explain how to introduce indels (NHEJ-mediated) or precise deletions (homology-dependent repair (HDR)-mediated) at precise targets. The second set of protocols describe how to swap a promoter or coding sequence to yield a reprogrammed gene. The methods do not require the use of dominant or auxotrophic marker genes and thus the strains generated are marker-free. The protocols have been tested in multiple *K. marxianus* strains, are straightforward and can be carried out in any molecular biology laboratory without specialized equipment.

Keywords: genome editing; Golden Gate assembly; non-conventional yeast; industrial biotechnology; strain engineering; synthetic biology

INTRODUCTION

Kluyveromyces marxianus is a yeast with much promise as a next-generation cell factory. Its thermotolerance, fast growth and diverse nutrient utilization abilities make it a good host for economical bio-based production. Developing a yeast species to be a cell factory requires regulatory parts for gene expression,

gene expression systems and tools for efficient genome engineering and genome-scale modeling for facilitating design. In the case of *K. marxianus*, all of these components have been, or are in the process of, being characterized, developed and made available to the research community (reviewed in Nurchoilis et al. 2020). Without doubt, the most important requirements for strain engineering are the capacity to efficiently inactivate

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specific genes and to introduce new alleles, variants or heterologous genes into defined chromosomal loci. As with many other organisms, the development of CRISPR/Cas9 genome engineering methods been a game-changer for the metabolic engineering of *K. marxianus*. At this point in time, five different Cas9 systems have been published for this yeast (Table 1), each of which uses slightly different variations of the theme (Löbs et al. 2017; Nambu-Nishida et al. 2017; Cernak et al. 2018; Juergens et al. 2018; Lee et al. 2018). Each system was developed and validated using specific target genes and different host strains, and there has not been any systematic comparison between them. Accordingly, it can be concluded that all methods work and the choice of system is largely dictated by ease of access to resources, available protocols and speed and reliability of the method. Our group developed one of these genome engineering tools: a single plasmid that carries SpCas9 as well as a gRNA expression cassette (Juergens et al. 2018; Rajkumar et al. 2019; Varela et al. 2019). In this system, the sgRNA is expressed from a constitutive RNA polymerase II promoter and liberated from the transcript with two ribozymes: a hammerhead (HH) ribozyme at the 5' end and a hepatitis delta viral (HDV) ribozyme at the 3' end. For more rapid and less expensive plasmid construction, the original pUDP002 plasmid (Juergens et al. 2018) was modified to allow the use of short oligonucleotides to introduce gRNA targets (Varela et al. 2019). Although this new plasmid, pUCC001, does not fully reform the base-pairing at the 5' and 3' ends of the HH ribozyme, cleavage still appears sufficient and we use this plasmid for routine and efficient introduction of double stranded breaks (DSB) in genomic targets. As an illustration of utility, this system was used for the inactivation of multiple sugar transporters in a single strain (Donzella et al. 2021), or the genetic reprogramming of an endogenous pathway with a combination of gene knock-outs, promoter swaps, allelic replacements and introduction of heterologous genes (Rajkumar and Morrissey 2020).

An important step in disseminating the tools and techniques for the genetic and metabolic engineering of *K. marxianus* is the provision of protocols to allow their rapid adoption. As the cloning of gRNA targets into pUCC001 is the simplest among published methods, a detailed step-by-step workflow and set of protocols for strain engineering in *K. marxianus* is provided here. These protocols provide for (i) constructing a gRNA plasmid targeting a single gene, (ii) performing single gene knock-outs in wild-type and non-homologous end-joining (NHEJ) deficient strains and (iii) carrying out marker-free promoter replacement to repress gene expression (Fig. 1). This latter method allows construction of 'knock-down' strains where expression of target gene is reduced or placed under the control of a regulated promoter. This is useful when dealing with essential genes. This third protocol can also be used for introduction of point mutations or allelic replacement. The methods have been tested in several commonly-used haploid and diploid *K. marxianus* strains.

Kluyveromyces marxianus has efficient non-homologous end joining (NHEJ) DNA repair and this is strongly favored over homology-dependent repair (HDR; Hoshida et al. 2014). Without the use of targeted DSB, it is difficult, though not impossible, to introduce DNA into specific targets as random genomic integration is favored. With CRISPR-mediated DSB, repair fragments can be introduced into targeted loci, though competition between NHEJ and HDR is still an issue and it is often necessary to screen quite a lot of transformants to identify correct integrants. This is overcome by using strains lacking NHEJ and we previously constructed and characterized *ku70* and *dnl4* (DNA ligase IV) mutants (Rajkumar et al. 2019). While both are effective, the *dnl4*

background is routinely used in this protocol and recommended for construction of gene deletion mutants. To this end a protocol for N-terminal deletion of the DNA ligase *Dnl4* in any host strain is also provided (Fig. 1). It is possible to introduce inactivating mutations using NHEJ in wild-type strains following the protocols provided simply by omitting the repair fragments. This method is effective but as mutations are typically SNPs or indels, they cannot be identified by PCR and candidate transformants must be sequenced. When such SNPs or indels are created, is not possible to precisely predict the mutation that will occur and if the resulting mutation is a single base change, there is the possibility of reversion. For these reasons, for stable gene inactivation, we recommend introduction of substantial deletions within the open reading frame (ORF) using a repair fragment. For routine gene disruptions, selectable markers are not required but repair fragments are easily modified if it is desired to use a dominant or auxotrophic marker. Starting from a known gene to be knocked out or replaced, a library of predicted gRNA targets is used to select optimal candidates to be cloned into pUCC001. Typically, 2–3 targets per gene should be tested as there still is no way of being certain that a particular target will work. pUCC001 is a modified version of pUDP002 that permits the cloning of a single gRNA target into the gRNA expression cassette by Golden Gate assembly using oligonucleotides for the target, whereas the parent plasmid required that the entire cassette be cloned anew for each gRNA target (Fig. 2). In parallel, a repair fragment to partially or completely delete the gene is designed from short annealing oligos and constructed by primer extension (Fig. 1). These are then co-transformed into *K. marxianus*, and transformant colonies screened for the deletion by a robust colony PCR protocol (Jakočiunas et al. 2015).

MATERIALS REQUIRED

Microbial strains

- Chemically competent *Escherichia coli* cells.
- The *K. marxianus* strain of choice, e.g. NBRC 1777, CBS 6556/ATCC 26 548, NCYC 587/ATCC 36907 or CBS 397. The strain may be wild-type or NHEJ deficient. In the case of the latter, this background can be created with CRISPR by a partial deletion of the DNA ligase IV *DNL4* as outlined below.

Plasmids and DNA

- Plasmid pUCC001 (Addgene #124451), containing SpCas9 and a gRNA expression cassette (Table 2).
- Primer Bsa-R (Table 3).
- For creating an NHEJ-deficient strain by inactivating *DNL4* (Table 3):
 - Primers ASR_J013NF and ASR_J014R for constructing the gRNA plasmid.
 - Oligonucleotides ASR_J013RF and ASR_J014RF for the repair fragment.
 - Primers ASR_J008F and ASR_J012NR for checking the a deletion in *DNL4*.
- Target-specific primers and oligos to be designed and ordered in the course of the protocol are not included; a list of these primers is provided in Table 4.

Culture media components

- Components for Lysogeny broth (LB), according to Miller.
- Components for YPD broth.

Table 1. Comparison of *K. marxianus* genome engineering systems.

Plasmid name	Expression of gRNA:promoter (pr)/terminator (t)	Release mechanism	gRNA cloning method	Cas nuclease used	Cas9 expression	Copy number	Reference	Addgene ID
pUCC001	ScTDH3pr/CYC1t	Modified hammerhead (5') + hepatitis delta viral (3') ribozymes	Golden Gate assembly	SpCas9D147Y P411T	Constitutive	Medium ^a	Varela et al. (2019)	124451
pUDP002	ScTDH3pr/CYC1t	Hammerhead (5') + hepatitis delta viral (3') ribozymes	Gibson assembly	SpCas9D147Y P411T	Constitutive	Medium ^a	Juergens et al. (2018)	103872
pIW601	KmRPR1pr or ScSNR52pr/tRNAGly	tRNA processing	Gibson assembly	SpCas9	Constitutive	Low ^b	Löbs et al. (2017)	98907
pKCas9	KmRNR2pr	Hepatitis delta viral ribozyme	Restriction-based cloning	SpCas9	Constitutive	Medium ^a	Cernak et al. (2018)	N/a
nCas9-CDA_Base	KmSNR5pr/SUP4t	RNA polymerase III processing	Restriction-based cloning	nCas9D10A-CDA_Base	Constitutive	Low ^b	Nambu-Nishida et al. (2017)	N/a
Cas9_Base	KmSNR5pr/SUP4t	RNA polymerase III processing	Restriction-based cloning	SpCas9	Constitutive	Low ^b	Nambu-Nishida et al. (2017)	N/a
(n/a—Cas9 integrated)	KmSNR52pr/SUP4t	RNA polymerase III processing	PCR and restriction-based cloning	SpCas9	Inducible (lactose)	n/a	Lee et al. (2018)	N/a

a—derived from an autonomously replicated sequence (ARS); b—derived from a native or synthetic ARS/centromere combination.

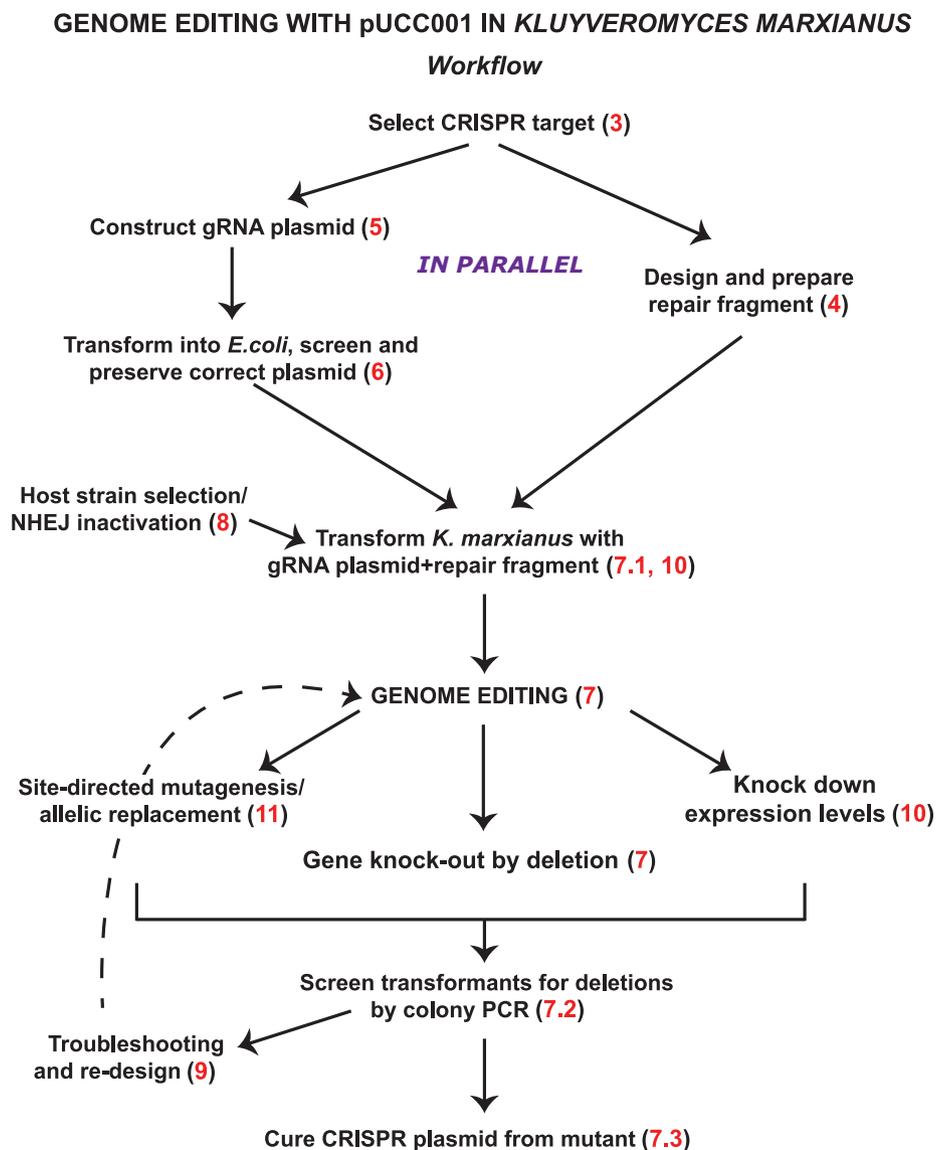


Figure 1. General workflow of the genome editing protocols with pUCC001. Sections in the text corresponding to different steps of the workflow are highlighted in red.

Table 2. List of plasmids to order.

Name	Function	Addgene ID	Reference
pUCC001	gRNA cloning plasmid	124451	Rajkumar et al. (2019); Varela et al. (2019)
Optional—for promoter knock-down			
YTK083	Plasmid backbone with ampicillin resistance	65190	Lee et al. (2015)
YTK030	GAL1 promoter from <i>S. cerevisiae</i>	65137	Lee et al. (2015)
pKmK.P8	REV1 promoter from <i>K. marxianus</i>	125041	Rajkumar et al. (2019)
pKmK.P10	GDH2 promoter from <i>K. marxianus</i>	125043	Rajkumar et al. (2019)

- Deionized water.
- Agar (AGR05, Formedium; Hunstanton, U.K.).
 - Depending on availability and costs, agar from any suitable supplier can be used.
- Alternatively, the following ready-made medium formulations can be used:
 - LB broth, Miller (LMM0102, Formedium).
 - YPD broth (CCM0210, Formedium).

Chemicals and antibiotics

- Deoxyribonucleic acid and sodium salt, from salmon testes (D1626, Sigma-Aldrich; Arklow, Ireland), henceforth, referred to as salmon sperm DNA.
- Polyethylene glycol (PEG) 3350 (88276, Sigma-Aldrich).
- Lithium acetate dihydrate (L4158, Sigma-Aldrich).

Table 3. List of primers.

Name	Sequence (5' to 3')	Use	Reference
Bsa-R	TACACGCGTTTGTACAGAAAAAAGAAAAATTTGA	Primer for verification of gRNA plasmid assembly by PCR and sequencing	Rajkumar et al. (2019)
		Optional—for DNL4 inactivation	
ASR_J013NF	CGTCAACCATTAACTCTGGAGA	Forward oligo for gRNA targeting DNL4	Nambu-Nishida et al. (2017); Rajkumar et al. (2019)
ASR_J014R	AAACTCTCCAGAGTTAAATGGTT	Reverse oligo for gRNA targeting DNL4	Nambu-Nishida et al. (2017); Rajkumar et al. (2019)
ASR_J013RF	TGTCAACTTCTAACAATGAGTGTTCCTGGTTCATGAGAAAAAG AACAGAAAGAATCACAACAATCTT	Left flanking repair fragment for DNL4	This work
ASR_J014RF	TGTTGGAGCTAGAAGGGTTT GTGTCCGAGTCTTCGGAATCTGAGATAATGACACAATGCTTT GATTAAGTGTTCCTTTTATATT	Right flanking repair fragment for DNL4	This work
ASR_J008F	ATAAACCCCTTCTAGCTCAA ATAAATCCATTATAAGGCAACT	Forward primer to check for DNL4 deletion	This work
ASR_J012NR	TGCGGAAACCTTCTGTATATAA	Forward primer to check for DNL4 deletion	This work
		Optional—for promoter knock-down	
ASR_K001F	TTTGCTGGCCTTTTGCTC	Colony PCR reverse primer for confirming knock-down repair fragment assembly	Rajkumar et al. (2019)
ASR_K001R	ATTGGTAACTGTACACCAAGTTTA	Colony PCR reverse primer for confirming knock-down repair fragment assembly	Rajkumar et al. (2019)

Table 4. List of primers to design and order.

Function	Overhangs	Suggested name
Forward oligo for gRNA	5'-CGTC-3'...	(gene)-gRNA.(target no.)_F
Reverse oligo for gRNA	5'-AAAC-3'...	(gene)-gRNA.(target no.)_R
Left flanking repair fragment	See Fig. 4, text	(gene)_RF_F
Right flanking repair fragment	See Fig. 3, text	(gene)_RF_R
Yeast colony PCR forward primer	None	(gene).chk_F
Yeast colony PCR reverse primer	None	(gene).chk_R
	Optional for promoter knock-down	
Forward oligo for promoter gRNA	5'-CGTC-3'...	(gene)pr.(target no.)_F
Reverse oligo for promoter gRNA	5'-AAAC-3'...	(gene)pr.(target no.)_R
Left homology arm forward primer	5'-ATCGGTCTCACCT-3'	(gene)_US_F
Left homology arm reverse primer	5'-ATCGGTCTCACGT-3'	(gene)_US_R
Right homology arm forward primer	5'-ATCGGTCTCATATG-3'	(gene)_DS_F
Right homology arm reverse primer	5'-CTCAGGTCTCATCGG-3'	(gene)_DS_R
Knock-down colony PCR forward primer	None	(gene).kd_F
Knock-down colony PCR reverse primer	None	(gene).kd_R
Promoter swap sequencing primer	None	(gene).seq_R

- An equivalent salt (98% purity or higher) from any other supplier may also be used.
- Molecular biology-grade water (W4502, Sigma-Aldrich).
- Glycerol, molecular biology grade (BP229-1, ThermoFisher).
 - Glycerol of an equivalent grade from other suppliers may be substituted.
- 100% or absolute ethanol (51976, Sigma-Aldrich).
- Ampicillin, sodium salt (BP1760-25, ThermoFisher).
- Hygromycin B (HYG1000/5000, Formedium).

Molecular biology enzymes and reagents

- OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (M0486S/L, New England Biolabs; Ipswich, U.S.A.).
 - Depending on availability and costs, DreamTaq Green PCR Master Mix (K1081/2, ThermoFisher; Carrigaline, Ireland) can be used as a substitute for OneTaq. Both enzymes have similar efficiencies and produce equivalent results for this protocol.

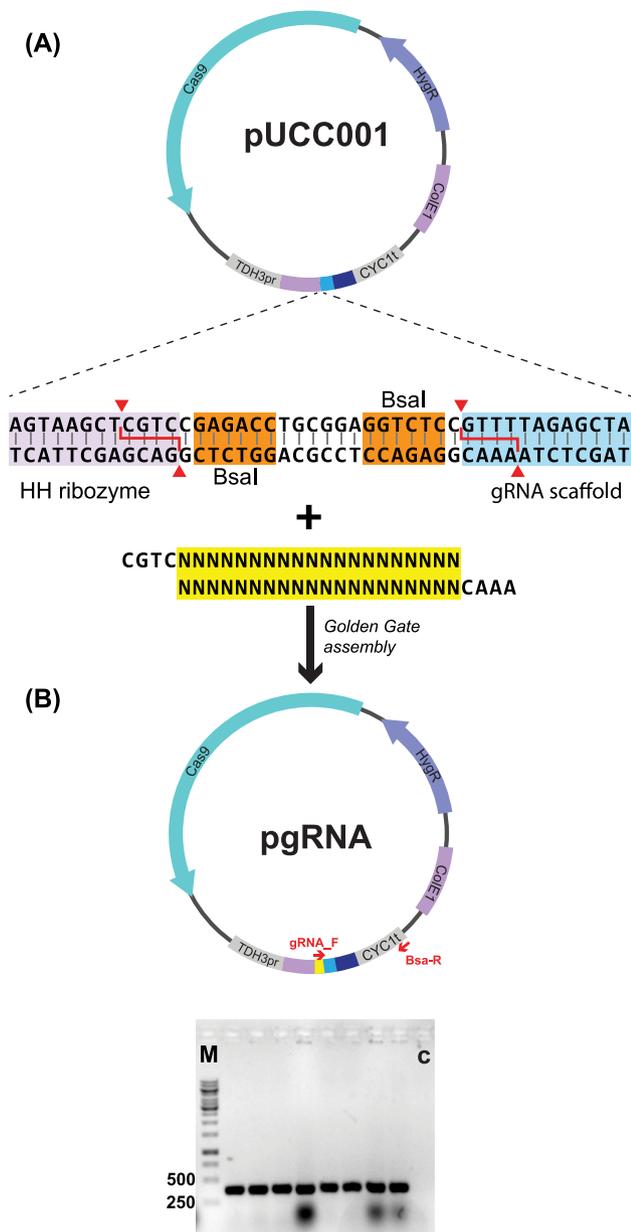


Figure 2. Combined Cas9 and gRNA expression system. (A) The combined Cas9/gRNA plasmid pUCC001 is compatible with multiple yeast species by virtue of a pangenomic ARS and cross-species expression systems. *Inset:* The ribozyme-based gRNA expression system has been modified to allow Golden Gate cloning of new gRNA targets from oligos containing the gRNA by creating BsaI cloning sites (marked in orange) downstream of the hammerhead (HH) ribozyme and upstream of the gRNA scaffold. (B) Annealed and phosphorylated oligos comprising the gRNA target are then cloned in by Golden Gate assembly to create a gRNA plasmid (pgRNA). The correct insertion of the target is verified by colony PCR, with the primers used marked on the plasmid. The representative agarose gel below shows confirmation of gRNA cloning by colony PCR, as seen by a band at ~380 bp. Colony PCR carried out on empty pUCC001 (C) does not yield a PCR product. The DNA marker (M) is the GeneRuler 1 kb ladder, with the bottom two bands' size indicated in bp. The image has been cropped and inverted for clarity.

- Q5 High-Fidelity 2X Master Mix (M0492S/L, New England Biolabs).
 - Depending on availability and costs, Phusion High-Fidelity Master Mix (New England Biolabs or ThermoFisher) can be substituted for Q5.
- 10x T4 DNA Ligase reaction buffer (B0202S, New England Biolabs)

- T4 polynucleotide kinase (M0201S/L, New England Biolabs).
- T7 DNA ligase (M0318S/L, New England Biolabs)
- BsaI-HFv2 (R3733S/L, New England Biolabs).
- Cytiva Illustra ExoProStar 1-Step (Cytiva US77702, ThermoFisher).
- GeneJET Plasmid Miniprep Kit (K0503, ThermoFisher).
- 50x Tris-acetate-EDTA (TAE) buffer (BP1332-1, ThermoFisher).
- Agarose for gel electrophoresis (BP160-500, ThermoFisher).
- GeneRuler 1 kb DNA ladder, ready-to-use (SM0313, ThermoFisher) or any equivalent DNA ladder covering 100 bp–10 kb.
- SafeView DNA stain (NBS Biologicals; Huntingdon, U.K.).
 - Alternatively any non-ethidium bromide DNA stain can be substituted in place of SafeView, depending on availability and costs.

Equipment and consumables needed

- PCR thermocycler with the ability to adjust temperature ramping.
- Benchtop microcentrifuge.
- Microcentrifuge tubes.
- Tabletop or standing centrifuge with a rotor that can accept 15- or 50-mL Falcon tubes.
- A Nanodrop (ThermoFisher) or similar spectrophotometer to measure DNA concentrations.
- Agarose gel electrophoresis set-up.
- Standard micropipettes (0.1–1 mL, 20–200 μ L, 2–20 μ L, 0.2–2 μ L or 0.5–10 μ L) with tips.
- Water bath.
- Heating block (shaking optional).
- 15 mL culture tubes or Falcon tubes.
- 50 mL Falcon tubes or Oak Ridge centrifuge tubes.
- PCR tubes, both single and strips.
- Spectrophotometer and cuvettes.

Bioinformatics tools

- Any program suitable for reading DNA sequences and designing DNA constructs and primers *in silico* (e.g VectorNTI, Clone-Manager, SnapGene, ApE or SerialCloner).

MEDIUM AND SOLUTION COMPOSITIONS

LB medium (Miller)

YPD medium

- Dissolve the following in 1 L deionized water:
 - Yeast extract 10 g.
 - Peptone 20 g.
 - D-glucose 20 g.
- The broth is autoclaved at 121°C for 15 min and stored at room temperature after autoclaving.
- For YPD agar, add 20 g/L agar after the components have dissolved and before autoclaving. Once the agar has cooled to the point that the bottle can be held bare-handed, pour into Petri plates. The plates are stored at 4°C.
- For YPD agar + hygromycin (YPD agar + hyg), once the agar has cooled to the point that the bottle can be held bare-handed, add 1 mL hygromycin stock solution (recipe below) per litre of agar. The antibiotic is mixed into the molten agar once cooled as above, and then poured into Petri plates. The plates are stored at 4°C and should be used within 3 months.

Single-stranded carrier DNA solution (carrier DNA/ssDNA)

50% (w/v) PEG 3350

- Weigh out 100 g of PEG 3350 in a graduated 250 mL beaker.
- Add deionized water slowly until the total volume of water + PEG in the beaker reaches 200 mL.
- Heat the solution and use a magnetic stirrer to dissolve. As the PEG dissolves, add more water to 200 mL.
- After the PEG has fully dissolved the volume is adjusted to 200 mL with deionized water for a final time after the stirrer bar has been removed.
- Transfer the solution to a 500 mL bottle and autoclaved at 121°C for 15 min. It is then stored at 4°C, making sure the bottle cap is kept tightly closed at all times.
 - Optionally, the PEG may be aliquoted into 15- or 50-mL Falcon tubes after autoclaving, and the tubes stored as above.

1M lithium acetate

80% (v/v) glycerol

- Measure out 160 mL of glycerol in a graduated beaker. Add 40 mL of deionized water, and the two liquids are mixed vigorously before transferring to a bottle.
- The solution is autoclaved at 121°C for 15 min and stored at 4°C.

2000× ampicillin solution (200 g/L)

- Dissolve 1 g of ampicillin in 5 mL of water.
- The solution is sterilized by filtration and dispensed into 1 mL aliquots that are stored at -20°C.

1000× hygromycin solution (200 g/L)

- Dissolve 1 g of hygromycin B in 5 mL of water.
- The solution is sterilized by filtration and dispensed into 1 mL aliquots that are stored at -20°C.

CRISPR TARGET SELECTION

1. If the target *K. marxianus* strain is NBRC 1777, a full list of predicted Cas9 targets is available at <https://doi.org/10.5281/zenodo.5554288> generated using sgRNAs9 (Xie et al. 2014).
2. If using Windows, the archive should be opened with a third-party software such as 7zip as it is too large for Windows Explorer's built-in archive reading function to open fully. The targets are organized by ORF ID and by chromosome number.
3. Identify the gene of interest's ORF ID based on the annotated genome available (for *K. marxianus* strain NBRC 1777) at https://www.ncbi.nlm.nih.gov/assembly/GCA_001417835.1/
4. Select and extract the folder corresponding to the gene from the main archive.
5. Open the file 'sgRNAs9_report.xls'. The prediction results should resemble those in Fig. 3.
6. Pick targets without off-target matches and a GC content below 60% and evaluated as 'Best' by the software. If something is known about the protein's functional domains or those of a homologue, pick a gRNA targeting the sequence coding for them.
7. gRNA targets containing the recognition site for BsaI (5'-GGTCTC-3') must be avoided.
8. After selecting 2–3 gRNA targets, order them as oligos for cloning into the Cas9/gRNA expression vector pUCC001. Order both the 20 nt target minus the PAM (the NGG at the end of the predicted targets) and its reverse complement (also omitting the PAM) with the following overhangs at their 5' ends: 5'-CGTC-3' for the forward primer and 5'-AAAC-3' for the reverse primer (Table 4). Design and order the oligos for the repair fragments, as well as the primers for PCR (see below in the section 'Design and Prepare Repair Fragment') along with the gRNA oligos.
9. If the target strain is not NBRC 1777, a list of predicted gRNA targets, conserved across eight *K. marxianus* strains, has also been compiled by another group and can be used to select a gRNA target if the strain of interest is one of those included in that study (Lee et al. 2018).
10. Alternatively, if the strain of interest is not included in the above list, the gRNA targets can be aligned against the gene of interest or BLASTed against other *K. marxianus* assemblies available at <https://www.ncbi.nlm.nih.gov/assembly/orfanism/4911/latest/>, depending on the sequence information available.

DESIGN AND PREPARE REPAIR FRAGMENT

1. Identify how much of the gene is to be deleted. Depending on the size of the gene targeted, the linear repair fragment used for deletion may span the entire gene, or a portion if the gene is large.
2. Select 85 bp long sequences flanking the gene or the region of the gene to be deleted. They need not start immediately upstream or downstream of the region to be deleted. The combined 170 bp sequence will form the repair fragment used for the deletion with the flanking sequences serving as homology arms.
3. The homology arms should be chosen so that all the gRNA targets selected in section III within the region flanked by them.
4. The fragment is to be generated by the primer extension of two 95-base oligonucleotides, each of which consist of the 85 bp homology arms + the last 10 bases of the other homology arm, as illustrated in Fig. 4.
5. Name the oligos (gene name)_RF_F and (gene name)_RF_R.
6. Design two primers flanking the homology arms for verification of the deletion by PCR. Ideally these should have an annealing temperature between 47 and 50°C for OneTaq using New England Biolabs' Tm Calculator at <https://tmcalculator.neb.com/#!/main>. The size of their amplicon should depend on the size of the gene to be deleted and the size of the gene. Name the primers (gene name)_chk.F and (gene name)_chk.R.
7. Set up the following reaction in a PCR tube for each gene to target with CRISPR:
8. Run the following reaction on the thermocycler after loading it with the tubes:
 - a. A total of 30 s initial denaturation at 98°C.
 - b. A total of 30 cycles of:
 - i. 10 s denaturation at 98°C.
 - ii. 20 s annealing at 55°C.
 - iii. 15 s extension at 72°C.
 - c. A total of 10 min final extension at 72°C.
 - d. Hold at 10°C.

sgRID	Start	End	Protospacer sequence+PAM(5'→3')	GC%	Pr	0M	1M	2M	3M	4M	5M	Total	No Seed	12+0M	1M	2M	3M	4M	5M	To	Risk evaluation	
77	KMAR_80308_S_87	1393	1415	GTGGCTCCAGCAGAAATCCAGG	55.0%	#	1	0	0	0	0	3	3#	1	0	0	0	0	0	0	0	Best
78	KMAR_80308_S_88	1409	1431	TTCCAGGCGAATCGTACTACTGG	50.0%	#	1	0	0	0	0	0	0#	1	0	0	0	0	0	0	0	Best
79	KMAR_80308_S_89	1410	1432	TCCAGGCGAATCGTACTACTGG	50.0%	#	1	0	0	0	0	0	0#	1	0	0	0	0	0	0	0	Best
80	KMAR_80308_S_90	1411	1433	CCAGGCGAATCGTACTACTGGG	55.0%	#	1	0	0	0	0	0	0#	1	0	0	0	0	0	0	0	Best
81	KMAR_80308_S_91	1427	1449	ACTGGGGTTACACTTCTTTCTGG	45.0%	#	1	1	0	0	0	2	3#	1	0	0	0	0	0	0	0	High_risk
82	KMAR_80308_S_92	1465	1487	CCAAACGTTGTTCCTCAACTGG	50.0%	#	1	0	0	0	1	1	2#	1	0	0	0	0	0	0	0	Best
83	KMAR_80308_S_93	1497	1519	CGACGTCGAGCTAGATTCCAAG	55.0%	#	1	0	0	0	1	2	3#	1	0	0	0	0	0	0	0	Best
84	KMAR_80308_S_94	1536	1558	GACTAACGATTACGAGAAGAAG	40.0%	#	1	0	0	0	0	3	3#	1	0	0	0	0	0	0	0	Best
85	KMAR_80308_S_95	1541	1563	ACGATTACGAGAAGAAGGTTG	40.0%	#	1	0	0	0	2	3	5#	1	0	0	0	0	0	0	0	Best
86	KMAR_80308_S_98	1578	1600	GTCATCAGATACAACAGCAAG	45.0%	#	1	0	0	0	1	2	3#	1	0	0	0	0	0	0	0	Best
87	KMAR_80308_S_99	1615	1637	CCTGTCGCTTTCGAATTAACAGG	45.0%	#	1	0	1	0	0	0	1#	1	0	0	0	0	0	0	0	Moderate_risk
88	KMAR_80308_A_3	1647	1669	CTGCGACTACATCTTCATCGTG	50.0%	#	1	0	0	0	0	2	2#	1	0	0	0	0	0	0	0	Best
89	KMAR_80308_A_4	1615	1637	CCTGTTAATTGCAAAAGCGACAG	40.0%	#	1	0	1	0	1	2	2#	1	0	0	0	0	0	0	0	Moderate_risk
90	KMAR_80308_A_5	1580	1602	TTCCCTTGCTGTGTATCTGATGG	40.0%	#	1	0	0	0	5	3	8#	1	0	0	0	0	0	0	0	Best
91	KMAR_80308_A_6	1522	1544	TCGTTAGCTTTAGCGATTACAG	40.0%	#	1	0	0	0	1	1	2#	1	0	0	0	0	0	0	0	Best
92	KMAR_80308_A_7	1514	1536	CTTTAGCGATTACAGGAGCCTTG	50.0%	#	1	0	0	0	2	1	3#	1	0	0	0	0	0	0	0	Best
93	KMAR_80308_A_8	1480	1502	ACGTCGTGTTTCAAACAGTTGG	45.0%	#	1	0	0	0	2	1	3#	1	0	0	0	0	0	0	0	Best

Figure 3. gRNA selection from genome-wide predicted targets. Typical results for gRNA target prediction from the sgRNAs9 predictions mentioned in the section 'CRISPR Target Selection' of the protocol. Some columns have been resized for clarity. Highlighted fields of interest are the gRNA target IDs for the sense (suffixed with .S) or antisense (suffixed with .A) ordered relative to the Start codon of the gene and its corresponding protospacer sequence including the PAM, GC content of the protospacer, the number of off-target matches in the genome for the protospacers containing 1-5 mismatches (columns 1-5 M) and finally the software's assessment of each target.

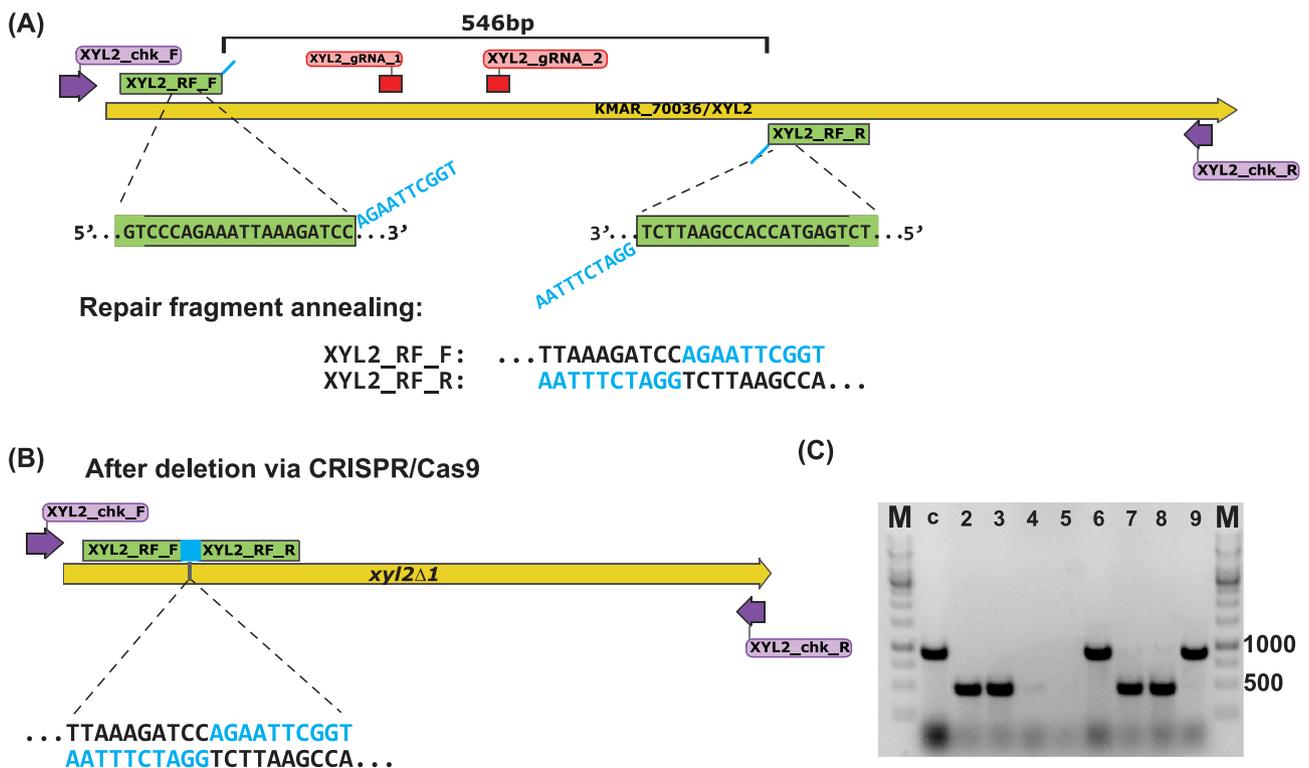


Figure 4. Design of a repair fragment for gene knock-out. The gene coding for xylitol dehydrogenase (XYL2) is used as an example. (A) Layout of gRNA targets, repair fragments (XYL2_RF_F/RF_R) and PCR verification primers (XYL2.chk.F/chk.R) for the XYL2 gene in *K. marxianus*. For this example a partial deletion of the gene was carried out. The 75 bp homology arm portions of the repair fragments are in green while the overhang parts of the repair fragments are in blue. The reverse complement of the last 10 bases of each homology arm are added to the 3' end of the opposite homology arm to create the oligonucleotides for repair fragment creation as shown by this close-up. The fusions form a 20 bp annealing region which are used for primer extension and synthesis by PCR. (B) Using Cas9, the repair fragments create an internal deletion 546 bp with the sequence at the deletion region corresponding to the annealing region of the fragment. (C) Confirmation of gene deletion by colony PCR. This is an example from an experiment where the intact gene amplified with the primers indicated in (A) gives a band of ~1 kb and a successful internal deletion, a band of ~500 bp. Colonies represented by lanes 2, 3, 7 and 8 carried the desired deletion in the target gene whereas those represented by lanes 6 and 9 retained the intact gene (lane 1). No bands were detected in lines 4 and 5 indicating a failure of the colony PCR.

9. If not used on the same day, the completed reaction can be stored at -20°C .

CONSTRUCTION OF THE gRNA PLASMID

1. Grow up the *E. coli* strain containing pUCC001 in 10 mL of LB + amp overnight and extract the plasmid using the GeneJet Miniprep Kit (Fisher Scientific; Carrigaline, Ireland) or any

suitable kit or protocol. The plasmid should be eluted in 30 μL of sterile water. The plasmid should ideally be at a concentration of 200–400 $\text{ng}/\mu\text{L}$.

- Dissolve the complementary gRNA insert primers in molecular biology-grade or autoclaved milliQ water (henceforth, referred to as sterile water) to a final concentration of 100 μM .
- Set up the following reaction for the phosphorylation and annealing of the primers:

Component	Volume (μL)
Q5 High-Fidelity 2X Master Mix	20
Repair fragment forward primer (100 μM)	2
Repair fragment reverse primer (100 μM)	2
Sterile H ₂ O	16
Total volume	40

Component	Volume (μL)
10 \times T4 DNA ligase buffer with ATP	1
Forward primer (100 μM)	1
Reverse primer (100 μM)	1
T4 polynucleotide kinase	1
Sterile H ₂ O	6
Total volume	10

- Incubate at 37°C for 30 min and then heat mixture to 95°C for 5 min. Finally ramp down to 25°C at a rate of 5°C/min.
- Dilute the phosphorylated and annealed primer mixture 1:200 with sterile water. This is the gRNA insert.
- Assemble the following reaction for a Golden Gate assembly (Fig. 2):

Component	Volume
10 \times T4 DNA ligase buffer with ATP (NEB)	1.5 μL
gRNA insert pUCC001	2 μL Enough for 100 ng
BsaI	0.5 μL
T7 Ligase (New England Biolabs)	0.5 μL
Sterile water	Upto 15 μL
Total volume	15 μL

- An aliquot of pUCC001 (from step 1) may be diluted as needed so as to have a volume of at least 0.5 μL to pipette for the reaction.
- Run the following program on a PCR thermocycler for the Golden Gate assembly:
 - A total of 25 cycles of:
 - 3 min at 37°C.
 - 4 min at 16°C.
 - 5 min at 50°C.
 - 5 min at 80°C.
 - Hold at 10°C.
- The reaction can be stored overnight at 4–10°C or for longer periods at –20°C.

CHEMICAL TRANSFORMATION INTO E. COLI AND VERIFICATION OF TRANSFORMANTS

Steps marked in *should be carried out aseptically, either under a laminar flow hood or around a Bunsen burner flame.

- Thaw out 20–30 μL of competent *E. coli* per gRNA plasmid on ice.
- At the same time, for each transformation, chill a sterile Eppendorf tube containing 15 μL of sterile water and 7.5 μL of the Golden Gate reaction on ice.
- When the cells have thawed out, they are to be stirred with a pipette tip to disperse them. Add 20–30 μL of competent cells to each Eppendorf tube, mix by flicking and incubate on ice for 20 min.
- *Heat shock for 30 s at 42°C in a water bath, then chill immediately on ice.
- *Add 300 μL of LB to each tube on ice.
- The tubes are closed and incubated at 37°C with shaking for 45 min.
- *Spread all of the transformation mix onto the surface of LB + amp plates. After the plates are dry, they are inverted and incubated at 37°C overnight (~16–24 h).
- The next day, select up to 6 colonies per plate for PCR screening.
- A small quantity of each colony is picked with a sterile pipette tip and resuspended in 2 μL of sterile water in a PCR tube.
- Prepare the following PCR mix. Multiply the quantity of each component by the number of colonies to screen + 2 (to account for pipetting errors and bubble formation):

Component	Volume (μL)
OneTaq Quick-Load	5
2xMaster Mix	
Forward gRNA (gene name).F (10 μM)	0.5
Bsa-R primer (10 μM)	0.5
Sterile H ₂ O	2

- For example, if eight colonies are to be screened, mix 50 μL OneTaq, 5 μL of each primer and 20 μL of water for the PCR mix.
- Transfer 8 μL of the PCR mix to each tube. Run the following program on the thermocycler:
 - A total of 6 min initial denaturation at 94°C
 - A total of 30 cycles of:
 - 30 s denaturation at 94°C.
 - 45 s annealing at 55°C.
 - 1 min extension at 68°C.
 - A total of 10 min final extension at 68°C.
 - Hold at 10°C.
- The annealing temperature in the above program has been found to work well for diverse gRNA plasmid constructions.
- A total of 5 μL of the PCR reaction are loaded on a 1% agarose gel stained with 0.1 μL SafeView/mL of gel and run for 30 min at 90 V to check PCR products. At least one well should be loaded with 3 μL of the ready-to-load GeneRuler 1 kb ladder on the gel. The BSA-R primer anneals in the CYC1 terminator of the gRNA expression cassette of pUCC001. A strong band around 380 bp (Fig. 2) indicates the presence of the gRNA insert in the plasmid.
- A total of two or three PCR-positive colonies are selected and grown overnight in 5 mL LB + 100 ng/ μL ampicillin at 37°C.

25. *Preserve 750 μL of each culture, mix them with 750 μL of 80% glycerol to be stored in a cryovial at -80°C as a stock.
26. The remaining culture is used to isolate the plasmid with the GeneJet Miniprep Kit (or any other appropriate kit or method) and their concentration measured on a Nanodrop. Concentrations using DH5 α plasmid-bearing strains usually are between 100–300 ng/ μL for the ~ 4 mL culture remaining using the GeneJet kit.
27. The gRNA cloned into the plasmid is verified by sequencing each plasmid using Bsa-R (Table 3) as a sequencing primer.
28. The stocks prepared in step 25 that contain sequence-verified plasmids are preserved as glycerol stocks.

GENOME EDITING OF A SINGLE GENE

Steps marked in *should be carried out aseptically, either under a laminar flow hood or around a Bunsen burner flame.

Transformation of *K. marxianus* with a gRNA plasmid and repair fragment

1. Isolate the gRNA plasmid(s) and pUCC001 if needed. A prep at a concentration of at least 300 $\mu\text{g}/\mu\text{L}$ is recommended.
 2. *Inoculate a single colony of the yeast strain of interest into 5 mL of YPD broth and incubate overnight at 30°C with agitation at 200 rpm.
 3. To break up clumps if they have formed. Dilute the culture 20- to 50-fold and measure its OD₆₀₀. Use water containing as much YPD as the diluted culture does for the blank.
 4. *Assuming a culture of volume of 10 mL per gRNA transformation, inoculate a flask containing YPD to a final OD₆₀₀ of 0.5. As *K. marxianus* is a more respiratory yeast than *Saccharomyces cerevisiae*, the culture should not take up more than 25% of the flask's volume.
 5. Enough culture should be grown for negative control (no plasmid) and no-gRNA/positive (pUCC001 alone) control transformations.
 6. Incubate the flask at 30°C with shaking for 3.5–4 h. This may be increased to 4–4.5 h if the strain to be transformed is a slow-growing mutant.
 7. If not done already, set up as many PCR reactions for generating the repair fragments required for the transformation as described in the section 'Construction of the gRNA Plasmid'.
 8. Thaw a tube of pre-denatured carrier DNA on ice prior to the next step.
 9. Harvest the cells by centrifugation at 5000 rpm for 5 min in a 50-mL centrifuge tube.
 10. *Resuspend the pellet in a volume of sterile water that is half of the culture volume. Centrifuge at 5000 rpm for 5 min at 20°C to pellet the cells. For cultures more than 50 mL, harvest the cells in two tubes.
 11. *Repeat the wash step.
 12. *Resuspend the cells in 1.0 mL of sterile water and transfer the cell suspension to a 1.5 mL microcentrifuge tube.
 13. Centrifuge for 30 s at maximum speed in a microcentrifuge and discard the supernatant.
 14. *The cells are resuspended in sterile water to a final volume of 100 μL per transformation. For example, given a culture grown for six transformations (step 4, 60 mL culture) the cells should be resuspended to a final volume of 600 μL .
 15. *Dispense the aliquots (a little over 100 μL each) in new microcentrifuge tubes, one for each transformation.
16. Make up sufficient transformation mix for the planned number of transformations, plus one extra, using the recipe below:
- | Component | Volume (μL) |
|---------------------|--------------------------|
| 50% PEG 3350 | 240 |
| 1 M lithium acetate | 36 |
| 2 mg/mL carrier DNA | 50 |
| Total volume | 326 |
17. For example, for five transformations, mix 1440 μL PEG 3350, 216 μL lithium acetate and 300 μL carrier DNA.
 18. Vortex this mixture vigorously until it is homogenous (usually 30 s) and store on ice.
 19. Centrifuge the tubes containing the cell aliquots maximum speed for 30 s in a microcentrifuge and the supernatant.
 20. *Add 326 μL of transformation mix to each tube.
 21. *Add 500 ng of gRNA plasmid to each tube (the volume will vary based on concentration, which should be in the range mentioned in step 2). If the volume added is x μL , $(34-x)$ μL of the repair fragment reaction should be added so that the final volume is 360 μL .
 22. *Resuspend the cells completely in the mix by vortexing and pipetting until no clumps of cells remain.
 23. Heat-shock the cells for 40 min at 42°C on a water bath.
 24. Centrifuge the tubes in a microcentrifuge at maximum speed for 30 s and completely remove the supernatant by pipetting.
 25. *The cells in each tube are then resuspended 1 mL of YPD by pipetting.
 26. Close the caps and incubate the tubes at 30°C for 2.5 h with shaking.
 27. Approximately, 1.5 h into the incubation, retrieve as many YPD + hygromycin plates as needed for the transformation from cold storage and prewarm them at 30°C .
 28. *Centrifuge the tubes at maximum speed for 30 s in a microcentrifuge and discard 600 μL of the supernatant. The cell pellet is resuspended in the remaining YPD and spread onto YPD + hygromycin plates. It is recommended to plate 300 μL neat and use the remainder for 1/5 and 1/10 dilutions (note: depending on the gene being deleted, the dilutions may not be necessary).
 29. Let the plates dry in a laminar flow hood to absorb the liquid, then invert them and incubate at 30°C for 3–5 days. Depending on the gene being deleted, colonies may take longer to appear.

Screening of colonies for deletions

1. Once colonies have appeared, select up to 10 colonies from each plate for screening by colony PCR.
2. If the gene deletion is known to impart a phenotype (e.g. inability to consume a carbon or nitrogen source, auxotrophy and sensitivity to a chemical), they may be pre-screened by counter-selection against this phenotype to narrow down the number of colonies of interest. This may be done by re-streaking or replica plating on the relevant medium. Colonies exhibiting the phenotype of interest can be selected for colony PCR.
3. Pipette 2 μL of sterile water into as many PCR tubes (or PCR tube strips) are required for the colonies to be screened.

4. Using a sterile 10 μL pipette tip, a small amount of each colony is picked and briefly resuspended in a PCR tube. The resultant suspension should be slightly cloudy and not opaque.
5. Close the tubes tightly and run the following lysis program on a thermocycler:
 - a. 30 s at 65°C.
 - b. 30 s at 8°C.
 - c. 1 min 30 s at 65°C.
 - d. 3 min at 97°C.
 - e. 1 min at 8°C.
 - f. 3 min at 65°C.
 - g. 1 min at 97°C.
 - h. 1 min at 65°C.
 - i. 10 min at 80°C.
 - j. Hold at 10°C.
6. While the program is running, make up the following mix on ice. Enough is to be prepared for the number of colonies to be screened + 2 (to account for pipetting errors and bubble formation):

Component	Volume (μL)
OneTaq Quick-Load 2 \times Master Mix	7.5
Forward primer (gene name).chk.F (10 μM)	0.75
Reverse primer (gene name).chk.R (10 μM)	0.75
Sterile H ₂ O	4

7. For example, if eight colonies are being screened, the mix will require 75 μL OneTaq, 7.5 μL forward primer, 7.5 μL reverse primer and 40 μL water.
8. All the reagents can be thawed at room temperature but kept on ice subsequently.
9. A colony that either comes from a no-gRNA control transformation or from the parent strain used in the transformation should be included in the screening so as to have a PCR product for the native gene for comparison.
10. If the transformation involved gRNAs targeting different genes, a separate mix for each gRNA and primer pair should be made.
11. After taking out the PCR tubes from the cyclor when the protocol in step 6 is over, directly add 13 μL of the mix and run the following program on the thermocycler:
 - a. A total of 6 min initial denaturation at 94°C.
 - b. A total of eight cycles of:
 - i. 35 s denaturation at 94°C.
 - ii. 45 s annealing at 60°C, with the temperature decreasing by 1°C every cycle.
 - iii. 2 min 30 s extension at 68°C.
 - c. A total of 32 cycles of:
 - i. 35 s denaturation at 94°C.
 - ii. 45 s annealing at 52°C.
 - iii. 2 min 30 s extension at 68°C.
 - d. 10 min final extension at 68°C.
 - e. Hold at 10°C.
12. The times and temperatures of each step in the cycle are robust and work for products between 200 and 2500 bp. If the expected PCR products (both for an intact or deleted gene) are \leq 1 kb, the extension time may be shortened to 1 min 30 s.
13. A total of 7.5 μL of the PCR reaction are run on a 1% agarose gel stained with 0.1 μL SafeView per mL of gel for 40 min at 90 V to check PCR products. At least one well should be loaded with 4 μL of the ready-to-load GeneRuler 1 kb ladder on the gel.
14. A gene deletion efficiency of at least 50% can be expected depending on the gRNA target and the size of the deletion.
15. Preserve two colonies whose PCR products show a deletion the predicted size for each gene targeted in this transformation.

Curing the CRISPR plasmid from mutants

1. *The colonies selected as above are grown in 2 mL of YPD for 24h at 30°C with shaking.
2. *The culture is then vortexed and passaged into 2 mL of fresh YPD at a 1:100 dilution. This fresh culture is grown for 24 h at 30°C with shaking.
3. *The culture is vortexed until any cell clumps or pellets are dispersed, streaked out onto both a YPD agar plate and a YPD + hygromycin plate to get single colonies. Incubate at 30°C until single colonies start to appear.
4. Optional: passage the yeast a third time into fresh YPD before streaking.
5. If colonies appear on the YPD + hygromycin plates, repeat steps 1–3.
6. *If colonies only appear on the YPD agar plates, inoculate a single colony from this plate and grow it overnight in 2 mL of YPD at 30°C with shaking
 - a. Optional: cells may be plated by serial dilution instead of a streak to make sure more single colonies are recovered from the cured culture. Inoculate the overnight culture from these. In the case of a diploid strain being edited, the colony PCR may be repeated on single colonies from the highest dilution where growth was seen. This is important to confirm that both copies of the gene were knocked out.
7. *Mix 500 μL of each culture with 500 μL of sterile 80% glycerol in a cryovial to homogeneity. Store the vial at -80°C .

Notes

1. While this protocol assumes the deletion is carried out in an NHEJ-deficient strain, the deletions can also be carried out in a strain with its NHEJ mechanisms intact if later experiments require it. The frequency of a successful deletion can decrease by a factor of 2–10 depending on the gene being targeted and the gRNA used.
2. Alternatively, this protocol can be used without repair fragments in a strain with intact NHEJ; in this case colony PCR products will need to be screened in turn by sequencing. It is not recommended as SNPs are more likely to revert and deletions are not guaranteed to take place in the PAM target.
3. The colony PCR protocol has been tested to work with other Taq and Taq blends and any suitable alternative to OneTaq may be used if necessary.
4. Depending on the size of the deletion attempted, it is recommended to isolate genomic DNA from both picked colonies and a no-gRNA control colony for checking a deletion. The efficiency of the colony PCR protocol outlined above decreases for amplicons $>$ 2.5 kb. If the product for the control, unedited gene is beyond this limit, a PCR from

genomic DNA is recommended to avoid potential false positive results.

CREATION OF A NHEJ DEFICIENT STRAIN BY INACTIVATION OF DNL4

1. The primers required for inactivating *DNL4*, mentioned in the Materials section, are listed in Table 3 for ordering. All sequences have been verified to work in multiple *K. marxianus* strains (NBRC 1777, CBS 6556/ATCC 26548, CBS 397 and ATCC 36 907/NCYC 587).
2. Once ordered, the previous portions of this protocol are to be followed to construct the *dnl4Δ1* mutation. The gRNA in Table 3 targets the sequence coding for the N-terminus of *Dnl4p* and the repair fragment deletion results in a truncation of the first 219 bp of *DNL4* (or the loss of the protein's first 72 residues) plus a few bases upstream. This is reflected as a change in PCR product size: 1585 bp vs. 1823 bp for an intact gene.
3. The frequency of the deletion appearing in colonies is between 1/8 and 1/20 depending on the strain used.

TROUBLESHOOTING

1. **The gRNA plasmid does not contain the insert upon sequencing even though the colony PCR results were positive.** Replace the T4 ligase buffer in use with a fresh aliquot for the phosphorylation and Golden Gate assembly, as both steps' efficiency is dependent on ATP in the buffer. If the buffer being used is recent, the Golden Gate assembly should be repeated with a fresh dilution of the gRNA insert as an incorrect or absent dilution can result in more non-specific assembly.
2. **Poor yeast transformation efficiency.** While transformation efficiency is strain-dependent in *K. marxianus*, the freshness of the denatured carrier DNA can also influence efficiency. The tube of carrier DNA currently in use should be denatured again, or substituted with fresh aliquot of denatured carrier DNA from a stock. If not done before, 'no-gRNA' control transformation using pUCC001 should be included to set a baseline for colony numbers. Low colony numbers relative to this could also imply the inactivated gene causes a lethal or sick phenotype, discussed below. If the problem persists, it may be necessary to work through the troubleshooting advice given in the original lithium acetate/PEG transformation protocols (Gietz and Schiestl 2007).
3. **Poor genome editing efficiency.** Low genome editing efficiency can arise due to:
 - a. A sub-optimal gRNA, in which case others may need to be picked from the predicted targets.
 - b. The gene inactivation causes a phenotype causing poor growth. If deleting a homologue of this gene in *S. cerevisiae* or *Kluyveromyces lactis* is known to create such a phenotype, it may be possible to improve editing efficiency by carrying out steps 25–28 in the main protocol in alternate rich medium that avoids stressful conditions arising from the phenotype. For example, if a gene like *PDC1* involved in fermentation is being knocked out, the above-mentioned steps can be carried out using ethanol or glycerol as a carbon source instead of glucose. If the phenotype is lethal, the gene's expression may be knocked down instead of knocking it out, discussed below.

- c. The intended deletion is too large for efficient deletion. Design new repair fragments deleting a portion of the gene that will still inactivate it.
 - d. The repair fragment targets a repetitive region. Change one or both homology arms of the fragment to target a different region in or near the gene.
4. **Creation of an unexpected lethal or sick phenotype.** It is possible that a targeted gene that cannot be edited is essential either by itself or in combination with existing mutations in the strain being used. In such a situation, it may be necessary to knock-down (i.e. decrease below native levels) expression using a weak or repressible promoter as described below.

PROMOTER KNOCK-DOWN OF A GENE BY CRISPR

Extra materials required

- Yeast toolkit (YTK) plasmid YTK083 (Addgene #65190) for a backbone (Lee et al. 2015).
- YTK plasmid YTK030 (Addgene #65137) for a repressible promoter swap (Lee et al. 2015).
- Plasmid pKmK.P8 (Addgene #125041) or pKmK.P10 (#125043) for a weak promoter swap (Rajkumar et al. 2019).
- Chloramphenicol (Sigma-Aldrich C0378-25G).
 - Prepare a 1000× stock by dissolving 250 mg of chloramphenicol in 5 mL of ethanol. Dispense into 1 mL aliquots and store at –20°C.
- GeneJet PCR Purification Kit (ThermoFisher, K0702)
- NotI (ThermoFisher, FD0594).
- Galactose (ThermoFisher, 10011560).
- Cytiva Ilustra ExoProStar 1-Step (ThermoFisher, 12124082).
- YPGal medium: prepared as with YPD, but with galactose substituted for glucose.
- YPGal agar plates.
- YPGal agar + hygromycin plates.
- Primer ASR.K001F: 5'-tttgctggcctttgtctc-3'.
- Primer ASR.K001R 5'-attgtaactgtcagaccaagtta-3'.

Identification of a target gRNA

1. As the set of predicted gRNA targets is limited to *K. marxianus* ORFs alone, a gRNA targeting a promoter must first be identified manually.
2. Copy the promoter region for the gene of interest, usually the first 600 bp upstream of its start codon.
3. Use the CHOPCHOP tool to predict gRNA targets using the 'Paste Target' option, as described in Labun et al. (2021).
4. Select a number of predicted gRNA targets and run a BLAST search with them as queries against the *K. marxianus* genome to check for off-target matches and pick two that have none.
5. Construct a gRNA plasmid as described in the sections 'Construction of the gRNA Plasmid' and 'Chemical Transformation into *E. coli* and Verification of Transformants' and verify the insert by sequencing.

Construction of the repair fragment

Steps marked in *should be carried out aseptically, either under a laminar flow hood or around a Bunsen burner flame.

1. Design primers to amplify 500 bp homology arms upstream and downstream of the promoter to be replaced (Fig. 5A).

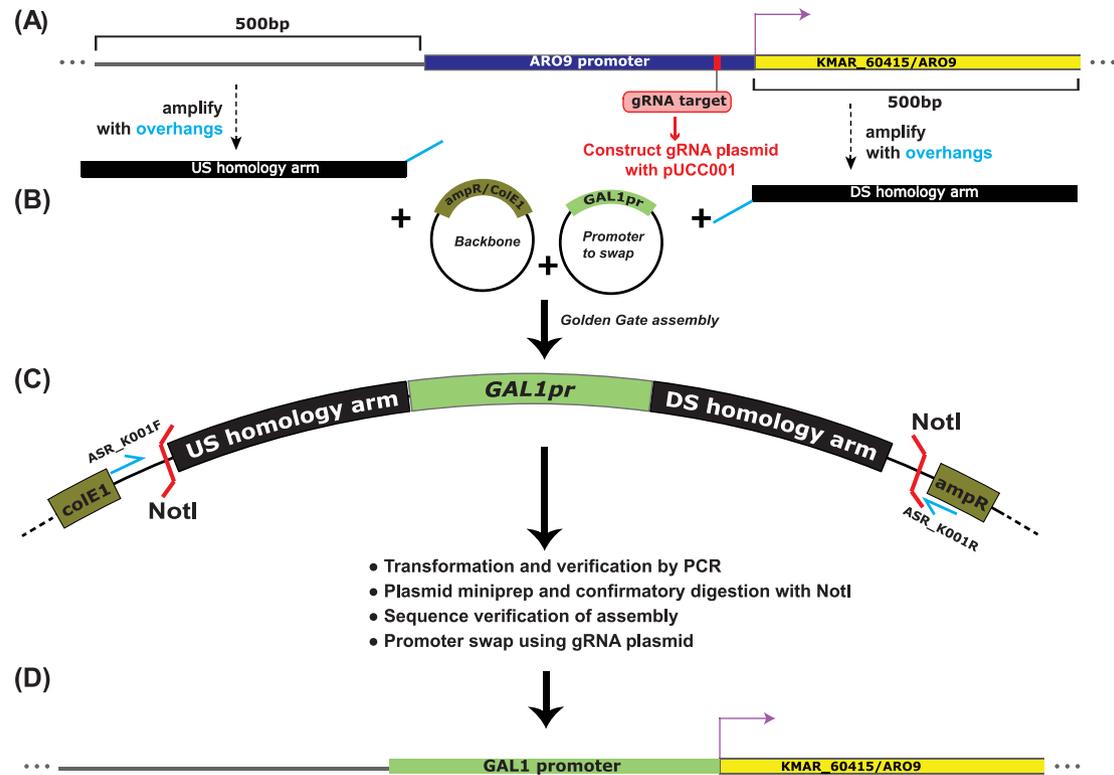


Figure 5. Construction of the repair fragment plasmid for knock-down. The gene coding for the aromatic aminotransferase II (ARO9) is used as an example. (A) The homology arms for the repair fragment are selected from the sequence surrounding the promoter (in blue) to be replaced. The downstream arm always incorporates the first 500 bp of the gene of interest. At the same time, a gRNA target within the promoter (in red) is selected as described in 'Promoter Knock-Down of a Gene by CRISPR' by examining the promoter sequence, and this is used to construct a gRNA expression plasmid with this sequence as described in 'Construction of the gRNA Plasmid' and 'Chemical Transformation into *E. coli* and Verification of Transformants'. (B) The arms are amplified with overhangs with BsaI sites as described in the text. These are used to construct the repair fragment plasmid by Golden Gate assembly with the YTK-compatible plasmids bearing the substituting promoter and plasmid backbone with an *E. coli* marker and origin. (C) Schematic zoomed-in view of the assembled repair fragment on the plasmid. Verification of correct plasmid assembly is carried out by colony PCR of *E. coli* into which the Golden Gate assembly was transformed, with the location of suitable primers marked (sequences provided in 'Promoter Knock-Down of a Gene by CRISPR'). The repair fragment is flanked by two NotI sites in the backbone for linearization. (D) During knock-down, Cas9 is used to introduce a DSB in the promoter region and thus the native promoter is seamlessly replaced with the ScGAL1 promoter using the linearised repair fragment plasmid. This method is easily adapted for allelic swaps and introduction of point mutations.

Match the melting temperatures of all primers so that they have the same calculated annealing temperature for Q5 High-Fidelity Master Mix according to the New England Biolabs' Tm Calculator at 500 nM primer concentrations.

- Add the following overhangs to each primer:
 - Left homology arm, forward primer: 5'-ATCGG TCTCACCT-3'.
 - Left homology arm, reverse primer: 5'-ATCGGT CTCAGTT-3'.
 - Right homology arm, forward primer: 5'-ATCGGT CTCATATG-3'.
 - Right homology arm, reverse primer: 5-CTCAGGT CTCATCGG-3'.
- The overhangs contain BsaI sites as well as overhangs based on the Yeast Toolkit system to directionally clone the parts into the repair fragment plasmid (Lee et al. 2015).
- If the arms contain any native BsaI sites near their ends (the 5' for the US arm or the 3' end for the DS arm) the homology arms can be shortened down to 300 bp, if needed to remove them, as these sites will interfere with Golden Gate assembly.
- If native BsaI sites cannot be removed from the homology arms, an alternate Golden Gate assembly protocol can be used.
- The right homology arm must consist of the first 500 bp of the gene of interest downstream of its start codon (the start

codon is contained in the overhang added by PCR) to ensure seamless integration of the promoter and its correct functioning.

- Design two more primers 50–100 bp away from the homology arms for PCR checking as well as a sequencing primer about 150 bp downstream of the gene's start codon to verify in-frame integration of the promoter. The same guidelines outlined in the sections 'Design and Prepare Repair Fragment' and 'Chemical Transformation into *E. coli* and Verification of Transformants' should be observed in designing these primers.
- Amplify each arm from *K. marxianus* genomic DNA isolated by any appropriate method using Q5 polymerase as follows:

Component	Volume (μ L)
Q5 High-Fidelity 2X Master Mix	20
Homology arm forward primer (10 μ M)	2
Homology arm reverse primer (10 μ M)	2
Genomic DNA, diluted as needed	1
Sterile H ₂ O	15
Total volume	40

9. Set up three reactions per homology arm.
 10. Run the following reaction on the thermocycler after loading it with the tubes:
 - a. A total of 30 s initial denaturation at 98°C.
 - b. A total of 30 cycles of:
 - i. 10 s denaturation at 98°C.
 - ii. 20 s annealing at 60°C
 - iii. 45 s extension at 72°C.
 - c. 10 min final extension at 72°C.
 - d. Hold at 10°C.
 11. Pool the reactions for each arm together and run 5 µL on a 1% agarose gel stained with 0.1 µL SafeView/mL of gel at 90 V for 40 min. At least one well should be loaded with the GeneRuler 1 kb ladder to confirm that the arm is amplified.
 12. Purify the PCR reactions for each arm with the GeneJet PCR Purification kit (or any equivalent kit or protocol) and measure their concentrations. A concentration of at least 30 ng/µL is recommended. Based on the promoter selected and the homology arms, a hypothetical map of the repair fragment plasmid should be constructed at this stage from the above sequences and the backbone from YTK083.
 13. Grow up cultures of plasmid YTK030 or pKmK.P8/pKmK.P10 in 5 mL of LB + 50 ng/µL chloramphenicol, as well as a culture of plasmid YTK083 in 5 mL of LB + 100 ng/µL ampicillin (LB + amp), both from stocks. Extract plasmids from them using the GeneJet MiniPrep kit. A typical yield is 100–200 ng/µL.
 14. Convert the concentrations of the plasmids and PCR products from ng/µL to nM: $Concentration (nM) = \left(\frac{1,000,000}{660}\right) \times \left(\frac{Concentration\ in\ ng/\mu L}{DN\ A\ molecule\ size\ in\ bp}\right)$
 15. A concentration of 1 nM corresponds to 1 fmol/µL.
 16. Assemble the following reaction for a Golden Gate assembly (Fig. 5B):
- | Component | Volume |
|--|--------------------|
| 10× T4 DNA ligase buffer with ATP (NEB) | 1 µL |
| Left homology arm | Enough for 40 fmol |
| Promoter of choice (YTK030, pKmK.P8 or pKmK.P10) | Enough for 40 fmol |
| Right homology arm | Enough for 40 fmol |
| YTK083 | Enough for 20 fmol |
| BsaI | 0.5 µL |
| T7 Ligase (New England Biolabs) | 0.5 µL |
| Sterile water | Upto 10 µL |
| Total volume | 10 µL |
17. The DNA of any part in the Golden Gate reaction may be diluted as needed so as to have a volume of at least 0.5 µL to pipette.
 18. Run the following program on a PCR thermocycler for the Golden Gate assembly:
 - a. A total of 25 cycles of:
 - i. 2 min at 42°C.
 - ii. 5 min at 16°C.
 - b. 10 min at 60°C.
 - c. 10 min at 80°C.
 - d. Hold at 10°C.
 19. If the homology arms contained native BsaI sites, steps (b) and (c) should be omitted from the protocol. This will allow the protocol to end with a ligation and avoid the native sites from being cut and mis-annealed again during the heating steps. However, this will result in an increased background during subsequent transformation into *E. coli*.
 20. *Transform 5 µL of the Golden Gate assembly into competent *E. coli* as described in the section 'Chemical Transformation into *E. coli* and Verification of Transformants' and spread the cells on LB + ampicillin agar plates.
 21. YTK083 has an RFP drop-out cassette; plasmids with an insert assembled into it will give rise to white colonies. Pick upto eight white colonies.
 22. Carry out a colony PCR as in the section 'Chemical Transformation into *E. coli* and Verification of Transformants' using primers ASR.K001F and ASR.K001R (Fig. 5C) and run the products on an agarose gel as before.
 23. Depending on the promoter used, the correct PCR product size should be:
 - a. YTK030/GAL1 promoter from *S. cerevisiae* (GAL1pr): 1587 bp.
 - b. pKmK.P8/KmREV1 promoter from *K. marxianus* (REV1pr): 2022 bp.
 - c. pKmK.P10/KmGDH2 promoter from *K. marxianus* (GDH2pr): 2017 bp.
 24. *Grow up 2–3 PCR-positive colonies overnight in 5 mL LB + amp.
 25. *Optional: to make sure a positive colony containing only the right plasmid is selected, the PCR-positive colonies can be streaked on fresh LB + amp plates and incubated overnight at 37°C. Single white colonies taken from these plates can be used to inoculate cultures.
 26. *Keep 0.75 mL of the culture aside and store it at 4°C. The remaining culture can be used to isolate the plasmid with the GeneJet MiniPrep kit (or equivalent kit or protocol). The yield should be between 100 and 200 ng/µL.
 27. Digest 100 ng of the plasmid with 0.5 µL FastDigest NotI at 37°C for 1 h. The backbone from YTK083 has two NotI sites flanking the insert for the purpose of linearization which can be used for a diagnostic digestion at this step. The FD Green buffer provided with the enzyme should be used since it comes pre-mixed with a loading dye for gel electrophoresis.
 28. Load the entire reaction on a 1% agarose gel and run it for 50–60min at 90 V with the GeneRuler 1 kb ladder.
 29. Unless the homology arms contain an internal NotI site, there should be two bands per digest: one at 1.86 kb (for the backbone) and one at ~2.4 kb (if using REV1pr or GDH2pr) or a single thick band at ~1.8–1.9 kb (if using GAL1pr; the single band is made up of backbone and insert bands that are too close to easily resolve) with the predicted size based on the hypothetical map.
 30. If none of the PCR-positive colonies yield a correct digest, more are to be screened.
 31. *Make a stock of a strain with the correct plasmid using the culture preserved earlier.
 32. It is recommended to sequence the plasmid to ensure the join between the promoter and downstream homology arm is seamless as it contains part of the gene of interest, matching the one in the hypothetical plasmid map. The homology arms are short enough that ASR.K001R can be used for this task.

Yeast transformation and promoter knock-down

Steps marked in *should be carried out aseptically where needed, either under a laminar flow hood or around a Bunsen burner flame.

- *Grow up *E. coli* strains (from glycerol stocks) and isolate repair fragment plasmids for the knock-down. Each knock-down requires at least 1.5 μg of digested plasmid.
- *Set up *K. marxianus* cultures for transformation as in section 'Genome Editing of a Single Gene: Transformation of *K. marxianus* with a gRNA plasmid and repair fragment'. Include cultures for a no-gRNA control with pUCC001 and a repair-fragment only (no gRNA plasmid) control.
- While the cells are growing after dilution (step 5 of the section 'Genome Editing of a Single Gene: Transformation of *K. marxianus* with a gRNA plasmid and repair fragment'), digest 1.5–2 μg of plasmid with 1 μL of NotI in a volume of 35 μL for 2 h.
- Carry out the rest of the transformation and as described in 'Genome Editing of a Single Gene: Transformation of *K. marxianus* with a gRNA plasmid and repair fragment' with the same amounts of gRNA plasmid and repair fragment.
- If replacing the promoter with *GAL1pr*, YPGal broth and agar must be used for the steps following the heat shock.
- Screen by yeast colony PCR as described with the relevant primers and visualizing the results on an agarose gel as before, but load 5 μL instead of 7.5 μL .
- Depending on the promoter used, a product showing a promoter replacement will be 2.1–2.2 kb for a swap with *GDH2pr* or *REV1pr*, or 1.7–1.8 kb for a swap with *GAL1pr*.
- Preserve the remaining PCR product for positive reactions showing a promoter replacement.
- Mix 5 μL of the PCR reaction with 2 μL of ExoPro Star 1-Step. Incubate at 37°C for 15 min followed by 15 min at 80°C to inactivate the enzyme.
- Send off the reaction for sequencing without further clean-up using the sequencing primer.
- Compare the sequencing results with the plasmid map constructed for the repair fragment and select colonies with promoter knock-down and the new promoter in-frame with the gene.
- *Grow the colonies up in YPD (YPGal if knocking down with *GAL1pr*) and cure the cultures of the gRNA plasmid as described in the section 'Genome Editing of a Single Gene: Curing the CRISPR Plasmid from Mutants'.
- When studying the effect of inactivating a gene knocked down with *GAL1pr*, carry out growth in YPD or any appropriate medium but with glucose as a carbon source.
- The gRNA target should include the sequence to be changed, or be as close to it as possible.
- While designing the repair fragments, the following guidelines should be observed:
 - Instead of spanning the region to be deleted, the homology arms should immediately flank the sequence to be changed.
 - The annealing portion of the oligos making up the repair fragment should contain the mutated base or bases.
 - Furthermore, the mutation(s) should disturb either the gRNA target or the immediately downstream of it so that Cas9 does not try to cut the mutated sequence again.
- Following the transformation with the gRNA plasmid and repair fragments, colonies should be screened both by PCR and sequencing.
- It is recommended that colonies with an identified mutation have the mutation resequenced again after curing of the gRNA plasmid to make sure the mutation is stable.
- To improve mutagenesis efficiency, especially in the case of altering protein sequence, codons flanking the amino acid residue to be mutated can be replaced with alternate codons in the repair fragment, provided the local sequence and codon usage allow it.

Allelic replacement of a gene

- The protocol for promoter knock-down ('Promoter Knock-Down of a Gene by CRISPR') can be modified for the efficient, marker-free allelic replacement of a gene. The modifications are listed below.
- The homology arms should cover the immediate 500 bp upstream and downstream of the ORF to be replaced and the substituting gene should be amplified or ordered with appropriate overhangs. The following overhangs for the homology arm and gene primers should be used:
 - Left homology arm, forward primer: 5'-ATCGG TCTCACCT-3'.
 - Left homology arm, reverse primer: 5'-ATCGGT CTCACATA-3'.
 - The last base immediately upstream of the gene to be deleted should be omitted from this primer to make sure the join between homology arm, substituting gene and Golden Gate retains the local sequence context for translation.
 - Substituting gene, forward primer: 5'-ATCGG TCTCATATG-3'.
 - Substituting gene, reverse primer: 5'-ATCGGT CTCAGGAT-3'.
 - Right homology arm, forward primer: 5'-ATCGG TCTCAATCC-3'.
 - Right homology arm, reverse primer: 5-CTCAGG TCTCATCGG-3'.
- The start codon can be omitted from the forward primer for the substituting gene as the *BsaI* overhang (c.) contains one.
- The arms and gene are amplified and purified as in 'Promoter Knock-Down of a Gene by CRISPR: Construction of the repair fragment'.
- The repair fragment plasmid is constructed as in 'Promoter Knock-Down of a Gene by CRISPR: Construction of the repair fragment' using YTK083 as a backbone.
- It is recommended that the substituting gene in the repair fragment plasmid be sequence-verified after Golden Gate

Notes

- Shorter homology arms can be added to the promoter of interest by PCR but will lead to an accompanying decrease in integration efficiency and a higher background of hygromycin-resistant colonies during a transformation.

VARIATIONS OF THE PROTOCOL

Site-directed mutagenesis

- Instead of knocking out or partially deleting a gene, the genome editing protocol can be used to replace a single nucleotide or a codon if extra guidelines are followed while selecting a gRNA target and designing the repair fragments.

assembly, transformation, screening and restriction digestion to ensure the join between the substituting gene and upstream homology arm is seamless so as to preserve the local sequence context.

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